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# Syntheses and biological activities of fluorescent-labeled analogs of acylpolyamine toxin NPTX-594 isolated from the venom of Madagascar Joro spider

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ABSTRACT

Acylpolyamine-type spider toxins are known to be potent and specific blockers against glutamate receptors (GluRs). The present study describes the syntheses and biological activities of several fluorescent-labeled analogs related to a Madagascar Joro spider toxin NPTX-594 to analyze visually the unknown interaction between spider toxins and GluRs.



## 1. Introduction

The glutamic acid is known as an excitatory neurotransmitter in the mammalian central nervous system (CNS), and it closely takes part in study and memory.[1] Moreover, it functions as a neurotransmitter in neuromuscular combination part in the arthropod such as the insects and the crustaceans.[2] Acylpolyamine toxins in spider venom are known to inhibit glutamatergic neuromuscular transmission of GluRs in the mammalian CNS or the arthropods.[3–6] Therefore, the spider toxins seem to be valuable to elucidate the mechanism of glutamatergic neurotransmission in the brain; however, the interaction between the toxins and GluRs has not been clarified yet.

NPTX-594 (**1**) (Fig. 1) is a neurotoxic acylpolyamine isolated from the venom of a Madagascar Joro spider, *Nephila madagascariensis*.[7] The toxin is comprised of four constituents, that is, 2,4-dihydroxyphenylacetic acid (Dhpa), asparagine (Asn), 4,8-diaza-1,12-dodecanediamine (Dada) and lysine (Lys). We had already chemically synthesized NPTX-594, and confirmed that the estimated structure shown in Figure 1 was correct.[8]

In order to confirm visually the specific binding of spider toxins to GluRs, we planned to synthesize several fluorescent-labeled analogs of NPTX-594 (Fig. 1). So far as we know, there are several variations of the terminal aromatic residue in natural spider toxins,[9] that is, the Dhpa,[10] 4-hydroxyphenylacetyl,[11] indole-3-acetyl[10] and 4-hydroxyindole-3-acetyl[10] residues are known as general aromatic constituents. Replacement of the Dhpa residue in JSTX-3 by the 1-naphtylacetyl residue did not cause significant loss of the biological activity.[12] On the other hands, we observed an interesting fact indicating that the deletion of the Dhpa residue results in a loss of the biological activity from a study on the structure–activity relationship of NSTX-3.[13] These facts suggest that the terminal aromatic residues mentioned above are replaceable with other aromatic ones, and are important requisite for the exhibition of biological activity. Furthermore, we demonstrated that the analog in which the Lys residue in NPTX-594 molecule was replaced with the *N*-(4-aminobutyl)glycine (Abg) residue as a Lys equivalent showed three times higher biological activity than NPTX-594.[14]

On the basis of many facts mentioned above, we designed and synthesized NPTX-594 analogs **2**–**5** labeled with the fluorophore such as the 7-hydroxycoumarin-3-carbonyl (Hcc) residue or the 7-hydroxycoumarin-4-acetyl (Hca) residue that was used as a substitute for the Dhpa residue, respectively. We also describe the results of cricket bioassay of these analogs.

## 2. Chemistry

At first, the preparation of protected Dada derivative **13** as the central polyamine part in designed compounds was carried out

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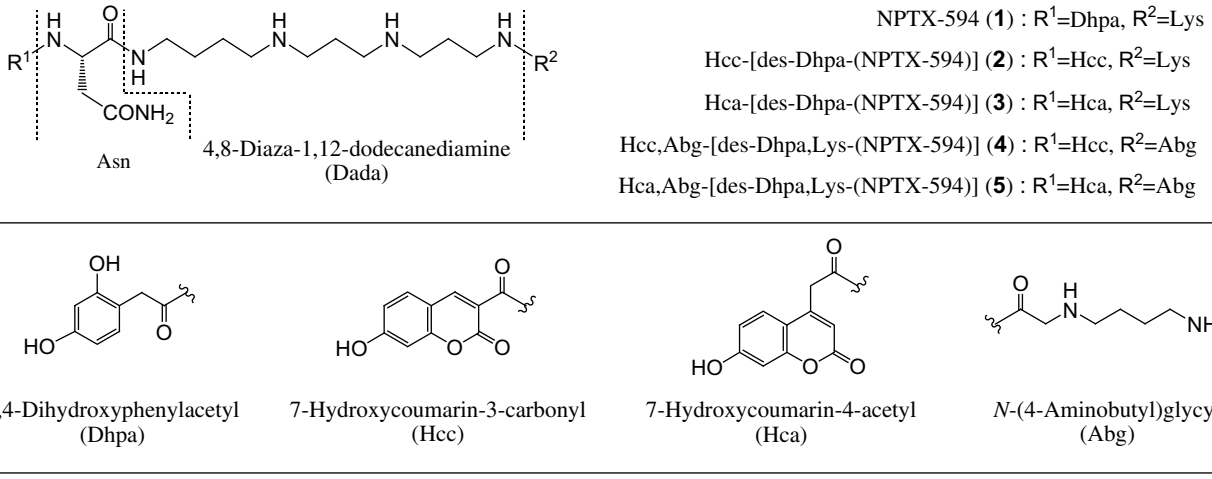
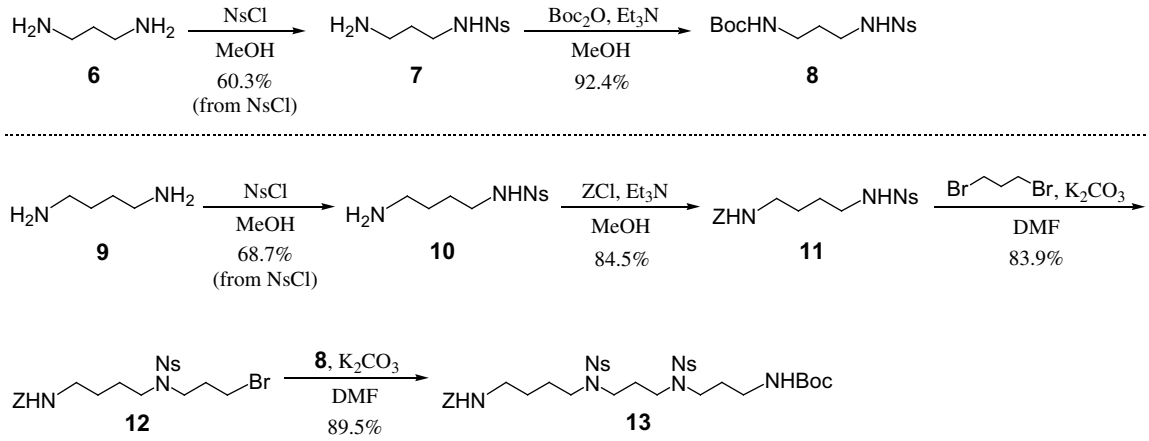



**Figure 1.** NPTX-594 (**1**) and its fluorescent-labeled analogs **2** –**5**.

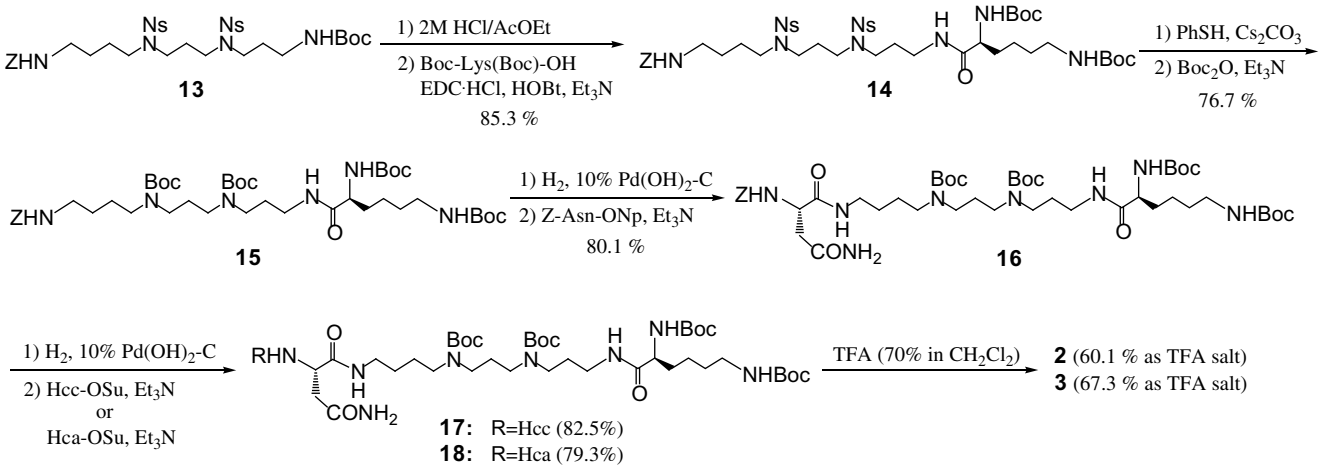



**Scheme 1.** Preparation of Dada derivative **13**.

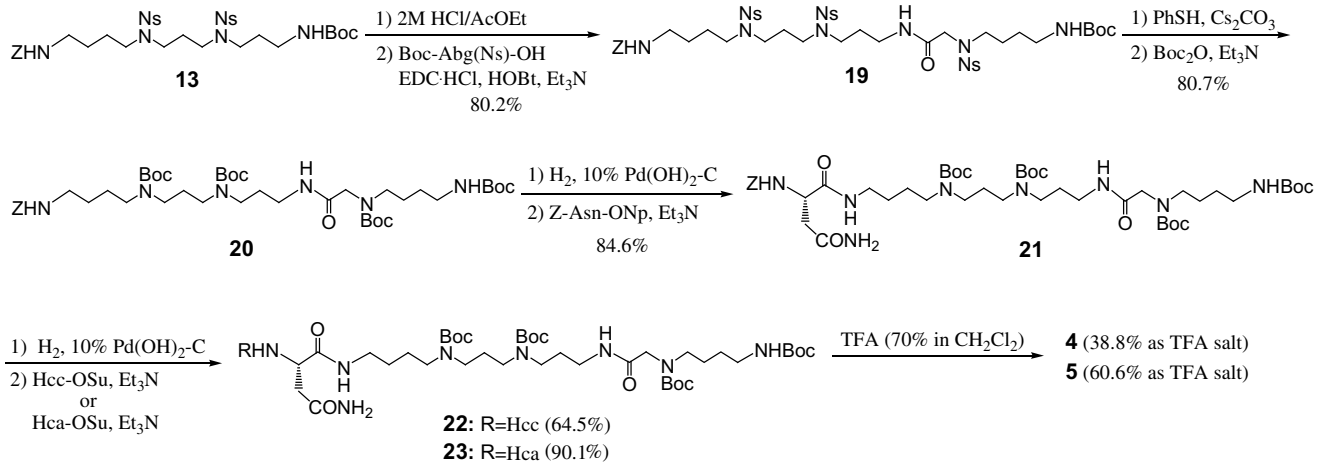
as shown in Scheme 1. In our previous study, the reductive N-alkylation method between primary amines and alkyl aldehydes was applied to prepare the polyamine residue,[8,14] however the yield of the reaction was not always good. Therefore, in the present paper we adopted the Fukuyama's method based on the coupling between alkyl halides and 2-nitrobenzenesulfonamides to improve the yield of N-alkylation.[15] 1,3-Propanediamine (**6**) was protected with the 2-nitrobenzenesulfonyl (Ns) and the *tert*-butoxycarbonyl (Boc) groups to give the compound **8**.[15] The protection of 1,4-butanediamine (**9**) with the Ns and the benzyloxycarbonyl (Z) groups was then carried out in a similar manner as the preparation of **8** to give the compound **11**, followed by the N-alkylation to Ns-amide with 1,3-dibromopropane according to the Fukuyama's method.[15] The bromide **12** thus obtained was then coupled with **8** to give the protected Dada derivative **13** in a similar manner as the preparation of **12**. After the removal of the *N*-Boc group of the compound **13**, the resulting free amino group was coupled with Boc-Lys(Boc)-OH by EDC·HCl/HOBt method (Scheme 2). The Ns groups of the product **14** were removed with thiophenol and $Cs_2CO_3$,[16] and the resulting free secondary amino groups were newly protected with the Boc group. The Z group of the compound **15** was removed by catalytic hydrogenation, and the Asn residue was introduced by the active ester method to give the compound **16**. The Z group of the compound **16** was removed again, and the resulting free amino group of the Asn residue was coupled with 7-hydroxycoumarin-3-carboxylic acid (Hcc-OH) or 7-hydroxycoumarin-4-acetic acid (Hca-OH) by the active ester method[17–19] to give the compound **17** or **18**, respectively. Finally, the Boc groups of the fully protected derivatives **17** and **18** were removed with TFA, and the crude products were purified by RP-HPLC to give the desired fluorescent-labeled analogs **2** and **3**, respectively. The syntheses of another analogs **4** and **5**, in which the Lys residue of original NPTX-594 was replaced with the Abg residue, were carried out in a similar manner as the syntheses of analogs **2** and **3** as shown in Scheme 3.

## 3. Results and discussion

The biological activities of the synthetic analogs **2–5** were evaluated by the paralytic assay against crickets (*Gryllus bimaculatus*) as reported in a previous paper,[14] and the results are summarized in Table 1. Of four synthetic analogs, the compounds **2** and **4** with the Hcc residue as an aromatic residue showed fairly lower activities than NPTX-594. On the other hand, the analog **5** with the Hca residue showed comparable activity to natural product, and the analog **3** showed less potent activity than the analog **5**. These results suggest that the Hca residue having methylene chain between coumarin skeleton and the carbonyl group seems to be a better substitute than the Hcc residue, since the former is structurally more analogous to the Dhpa residue than the latter. Furthermore, the $ED_{50}$ value of analog **5** suggests that the Abg




**Scheme 2.** Syntheses of fluorescent-labeled NPTX-594 analogs **2** and **3**.




**Scheme 3.** Syntheses of fluorescent-labeled NPTX-594 analogs **4** and **5**.

**Table 1**
Biological activities (cricket bioassay) of NPTX-594 and its fluorescent-labeled analogs

| Compounds | $ED_{50}$ (nmol/g) | 95% Confidence interval (nmol/g) |
|---|---|---|
| NPTX-594 (**1**) | 0.175 | 0.132–0.219 |
| **2** | 2.59 | 1.47–6.87 |
| **3** | 1.05 | 0.850–1.37 |
| **4** | 5.69 | 4.19–8.61 |
| **5** | 0.136 | 0.107–0.179 |

residue is a promising substitute for the Lys residue to develop any other NPTX-594 analogs with suitably potent biological activity in the future.

Further, we planned to examine the definitive inhibition activity of NPTX-594 or each fluorescent analog against GluRs by means of the patch-clamp method using the cells that are expressing the GluR genes is currently being undertaken.

## 4. Experimental

### 4.1. General

All of the melting points are uncorrected, and were measured by Yanaco MP-J3 (Yanaco Co., Ltd, Kyoto, Japan). Silica-gel column chromatography was carried out with silica gel PSQ 100B (Fuji Silysia, Aichi, Japan) at atmospheric pressure or with Merck silica gel 60 (#109385, 230–400 mesh, Merck, Germany) at medium pressure (1–5 kg $cm^{-2}$). $^1H$ NMR spectra were recorded on a Varian Mercury 300 (300 MHz, Varian Co. Ltd, USA) spectrometer. The chemical shifts in $^1H$ NMR are given in $\delta$ values from TMS used as an internal standard. Matrix assisted laser desorption ionization time of flight mass spectra (MALDI-TOF MS) were obtained on a KRATOS KOMPACT MALDI IV mass spectrometer (Shimadzu Co. Ltd, Kyoto, Japan). RP-HPLC was performed on Cosmosil $5C_{18}$-AR-II (10 × 250 mm, Nacalai Tesque Co. Ltd, Kyoto, Japan) for preparative purification. Elemental analyses were preformed using a MICRO CORDER JM10 (J-SIENCE LAB Co. Ltd, Japan). Z-Asn-ONp and 4 M HCl/AcOEt was purchased from Watanabe Chemicals Co. Ltd (Hiroshima, Japan).

### 4.2. Syntheses

#### 4.2.1. *N*-2-Nitrobenzenesulfonyl-1,3-propanediamine (7)

To a solution of 1,3-propanediamine (**6**) (5.00 g, 67.5 mmol) in MeOH (200 mL) was added 2-nitrobenzenesulfonyl chloride (NsCl) (4.98 g, 22.5 mmol) at 0 °C over a 35-min period, and the mixture was stirred for 24 h at rt. To the mixture was added NaOEt (1.53 g, 22.5 mmol), and the mixture was stirred for 1 h. The insoluble material was filtered off, and the filtrate was concentrated in vacuo. The crude product was purified by silica-gel column chro-

matography (Merck #109385, 100 g, $CHCl_3$/$Et_3N$/MeOH = 9:1:1) at medium pressure. The fractions containing the desired product were combined and concentrated in vacuo to give **15** as yellow oil (5.75 g, 60.3% from NsCl). $^1H$ NMR ($CDCl_3$) $\delta$ 1.62–1.71 (2H, m), 2.76–2.90 (4H, m), 7.72–7.75 (2H, m), 7.83–7.89 (1H, m), 8.12–8.15 (1H, m); MS Found *m*/*z* 260.2. Calcd for $C_9H_{14}N_3O_4S$: $[M+H]^+$, *m*/*z* 260.1.

#### 4.2.2. *N*$^1$-*tert*-Butoxycarbonyl-*N*$^3$-2-nitrobenzenesulfonyl-1,3-propanediamine (8)

To a solution of **7** (3.03 g, 10.3 mmol) in $CH_2Cl_2$ (50 mL) were added $Et_3N$ (3.59 mL, 22.5 mmol) and di-*tert*-butyl dicarbonate ($Boc_2O$) (3.36 mL, 14.0 mmol) at 0 °C, and the solution was stirred for 4 h at rt. The reaction mixture was washed with 10% citric acid (3× 15 mL) and brine (3× 15 mL). The organic layer was dried over anhydrous $MgSO_4$, and then concentrated in vacuo. The crude product was purified by silica-gel column chromatography (PSQ 100B, 60 g, hexane/AcOEt = 1:1). The fractions containing the desired product were combined and concentrated in vacuo to give **16** as yellow crystals (3.30 g, 92.4%). mp: 96.5–98 °C. $^1H$ NMR ($CDCl_3$) $\delta$ 1.42 (9H, s), 1.67–1.73 (2H, m), 3.12–3.25 (4H, m), 4.71 (1H, br s), 5.93 (1H, s), 7.72–7.87 (3H, m), 8.11–8.16 (1H, m). Anal. Found: C, 46.71; H, 5.96; N, 11.33%. Calcd for $C_{14}H_{21}N_3O_6S$: C, 46.79; H, 5.89; N, 11.69%.

#### 4.2.3. *N*-2-Nitrobenzenesulfonyl-1,4-butanediamine (10)

To a solution of 1,4-butanediamine (**9**) (2.64 g, 30.0 mmol) in MeOH (200 mL) was added 2-nitrobenzenesulfonyl chloride (NsCl) (2.20 g, 10.0 mmol) at 0 °C over a 35-min period, and the mixture was stirred for 24 h at rt. To the mixture was added NaOEt (1.53 g, 22.5 mmol), and the mixture was stirred for 1 h. The insoluble material was filtered off, and the filtrate was concentrated in vacuo. The crude product was purified by silica-gel column chromatography (Merck #109385, 60 g, $CHCl_3$/$Et_3N$/MeOH = 9:1:1) at medium pressure. The fractions containing the desired product were combined and concentrated in vacuo to give **10** as yellow oil (2.15 g, 68.7%, from NsCl). $^1H$ NMR ($CDCl_3$) $\delta$ 1.31 (2H, tt, *J* = 7.8, 6.8 Hz), 1.43 (2H, tt, *J* = 7.8, 6.8 Hz), 2.47 (2H, t, *J* = 6.8 Hz), 2.85(2H, t, *J* = 6.8 Hz), 4.25 (1H, br s), 7.80–7.87 (2H, m), 7.92–7.98 (2H, m); MS Found *m*/*z* 274.0. Calcd for $C_{10}H_{16}N_3O_4S$: $[M+H]^+$, *m*/*z* 274.1.

#### 4.2.4. *N*$^1$-Benzyloxycarbonyl-*N*$^4$-2-nitrobenzenesulfonyl-1,4-butanediamine (11)

To a solution of **10** (2.97 g, 11.5 mmol) in $CH_2Cl_2$ (15 mL) were added $Et_3N$ (5.11 mL, 36.7 mmol) and benzyloxycarbonyl chloride (ZCl) (3.07 mL, 13.9 mmol) at 0 °C. The mixture was stirred for 3 h at rt, and then concentrated in vacuo. The residue was dissolved in AcOEt (70 mL), and the solution was washed with 10% citric acid (3× 20 mL) and saturated aqueous $NaHCO_3$ (3× 20 mL). The organic layer was dried over anhydrous $MgSO_4$, and concentrated in vacuo. The thus-obtained crude product was purified by silica-gel column chromatography (PSQ 100B, 100 g, $CHCl_3$/MeOH = 15:1). The fractions containing the desired product were combined and concentrated in vacuo to give **11** as yellow crystals (4.35 g, 84.5%). Mp: 84.5–85 °C. $^1H$ NMR ($CDCl_3$) $\delta$ 1.56 (4H, br s), 3.10–3.18 (4H, m), 5.08 (2H, s), 7.35–7.36 (5H, m), 7.71–7.74 (2H, m), 7.84–7.87 (1H, m), 8.11–8.14 (1H, m); Anal. Found: C, 53.09; H, 5.45; N, 10.20%. Calcd for $C_{18}H_{21}N_3O_6S$: C, 53.06; H, 5.20; N, 10.31%.

#### 4.2.5. *N*$^1$-Benzyloxycarbonyl-4-(2-nitrobenzenesulfonyl)-4-aza-8-bromo-1-octylamine (12)

To a mixture of $K_2CO_3$ (2.58 g, 18.7 mmol) and 1,3-dibromopropane (3.25 mL, 31.9 mmol) being warmed at 60 °C under $N_2$ atmosphere was added a solution of **11** (2.00 g, 4.91 mmol) in anhydrous DMF (15 mL), and the mixture was stirred for 2 h at 60 °C under $N_2$ atmosphere. To the mixture was added water (15 mL), and the solution was extracted with diethyl ether (3× 20 mL). The extract was washed with 1 M HCl (3× 20 mL), saturated aqueous $NaHCO_3$ (3× 20 mL) and brine (3× 20 mL). The organic layer was dried over anhydrous $MgSO_4$, and concentrated in vacuo. The crude product was purified by silica-gel column chromatography (PSQ 100B, 65 g, benzene/AcOEt = 4:1). The fractions containing the desired product were combined and concentrated in vacuo to give **12** as yellow oil (2.31 g, 83.9%). $^1H$ NMR ($CDCl_3$) $\delta$ 1.50–1.65 (4H, m), 2.05–2.14 (2H, m), 3.16–3.23 (2H, m), 3.30–3.44 (6H, m), 4.78 (1H, br s), 5.09 (2H, s), 7.32–7.37 (5H, m), 7.60–7.70 (3H, m), 8.02–8.05 (1H, m); MS Found *m*/*z* 550.0. Calcd for $C_{21}H_{26}BrN_3O_6SNa$: $[M+Na]^+$, *m*/*z* 550.1.

#### 4.2.6. *N*$^{12}$-Benzyloxycarbonyl-*N*$^1$-*tert*-butoxycarbonyl-4,8-di(2-nitrobenzenesulfonyl)-4,8-diaza-1,12-dodecanediamine (13)

To a mixture of **12** (2.20 g, 4.16 mmol) and $K_2CO_3$ (1.73 g, 12.5 mmol) being warmed at 60 °C under $N_2$ atmosphere was added a solution of **8** (1.79 g, 4.99 mmol) in anhydrous DMF (27 mL), and the mixture was stirred for 23 h at 60 °C under $N_2$ atmosphere. To the mixture was added water (50 mL), and the solution was extracted with AcOEt (3× 40 mL). The extract was washed with 1 M HCl (3× 40 mL), saturated aqueous $NaHCO_3$ (3× 40 mL) and brine (3× 40 mL). The organic layer was dried over anhydrous $MgSO_4$, and concentrated in vacuo. The crude product was purified by silica-gel column chromatography (PSQ 100B, 70 g, benzene/AcOEt = 3:1). The fractions containing the desired product were combined and concentrated in vacuo to give **13** as yellow oil (2.95 g, 89.5%). $^1H$ NMR ($CDCl_3$) $\delta$ 1.43 (9H, s), 1.50–1.87 (8H, m), 3.10–3.34 (12H, m), 5.09 (2H, s), 7.31–7.37 (5H, m), 7.58–7.63 (2H, m), 7.67–7.71 (4H, m), 7.96–7.99 (2H, m); MS Found *m*/*z* 829.0. Calcd for $C_{35}H_{46}N_6O_{12}S_2Na$: $[M+Na]^+$, *m*/*z* 829.3.

#### 4.2.7. *N*$^{12}$-Benzyloxycarbonyl-*N*$^1$-[*N*$^\alpha$,*N*$^\varepsilon$-di(*tert*-butoxycarbonyl)lysyl]-4,8-di(2-nitrobenzenesulfonyl)-4,8-diaza-1,12-dodecanediamine (14)

To a solution of **13** (2.43 g, 3.01 mmol) in AcOEt (20 mL) was added 4 M HCl/AcOEt (20 mL), and the solution was stirred for 1 h at rt. The solvent was removed in vacuo to give colorless oil. The solution of the thus-obtained crude product in $CH_2Cl_2$ (35 mL) was neutralized with $Et_3N$, and then Boc-Lys(Boc)-OH (2.07 g, 4.52 mmol) HOBt·$H_2O$ (760 mg, 4.97 mmol), EDC·HCl (953 mg, 4.97 mmol) and $Et_3N$ (0.504 mL, 3.61 mmol) were added to the solution. The mixture was stirred for 20 h at rt, and then concentrated in vacuo. The residue was dissolved in AcOEt (100 mL), and the solution was washed with 10% citric acid (3× 30 mL), saturated aqueous $NaHCO_3$ (3× 30 mL) and brine (3× 30 mL). The organic layer was dried over anhydrous $MgSO_4$, and then concentrated in vacuo. The thus-obtained crude product was purified by silica-gel column chromatography (PSQ 100B, 80 g, hexane/AcOEt = 1:3). The fractions containing the desired product were combined and concentrated in vacuo to give **14** as yellow oil (2.85 g, 85.3%). $^1H$ NMR ($CDCl_3$) $\delta$ 1.37–1.63 (20H, m), 1.75 (2H, m), 1.86 (2H, m), 3.08–3.10 (2H, m), 3.16–3.32 (12H, m), 5.09 (2H, s), 7.33–7.36 (5H, m), 7.59–7.62 (2H, m), 7.76–7.71 (1H, m), 7.98–8.01 (1H, m); MS Found *m*/*z* 1057.4. Calcd for $C_{46}H_{66}N_8O_{15}S_2Na$: $[M+Na]^+$, *m*/*z* 1057.4.

#### 4.2.8. *N*$^{12}$-Benzylcarbonyl-4,8-di(*tert*-butoxycarbonyl)-*N*$^1$-[*N*$^\alpha$,*N*$^\varepsilon$-di(*tert*-butoxycarbonyl)lysyl]-4,8-diaza-1,12-dodecanediamine (15)

To a suspension of **14** (1.33 g, 1.25 mmol) in $CH_3CN$ (25 mL) were added $Cs_2CO_3$ (2.44 g, 7.48 mmol) and PhSH (0.307 mL, 2.99 mmol). The mixture was stirred for 15.5 h at rt. The solvent was removed in vacuo to give colorless oil. The oily residue was

dissolved in AcOEt (30 mL), and the solution was once dried over anhydrous $MgSO_4$, followed by concentration in vacuo. To the solution of the thus-obtained crude product in $CH_2Cl_2$ (2.5 mL) were added $Et_3N$ (0.765 mL, 5.49 mmol) and $Boc_2O$ (817 mg, 3.74 mmol), and the solution was stirred for 21 h at rt. The solvent was removed in vacuo, and the residue was dissolved in AcOEt (100 mL). The solution was washed with 10% citric acid (3× 30 mL), saturated aqueous $NaHCO_3$ (3× 30 mL) and brine (3× 30 mL). The organic layer was dried over anhydrous $MgSO_4$, and concentrated in vacuo. The crude product was purified by silica-gel column chromatography (PSQ 100B, 20 g, benzene/AcOEt = 1:1). The fractions containing the desired product were combined and concentrated in vacuo to give **14** as colorless oil (833 mg, 76.7%). $^1$H NMR ($CDCl_3$) $\delta$ 1.22–1.38 (2H, m), 1.37–1.50 (38H, s), 1.64–1.84 (8H, m), 3.08–3.36 (14H, m), 3.99–4.16 (1H, m), 5.09 (2H, s), 7.33–7.36 (5H, m); MS Found *m*/*z* 887.7. Calcd for $C_{44}H_{76}N_6O_{11}Na$: $[M+Na]^+$, *m*/*z* 887.5.

#### 4.2.9. $N^{12}$-($N^{\alpha}$-Benzyloxycarbonylasparaginyl)-4,8-di(*tert*-butoxycarbonyl)-$N^1$-[$N^{\alpha}$,$N^{\varepsilon}$-di(*tert*-butoxycarbonyl)lysyl]-4,8-diaza-1,12-dodecanediamine (16)

To a solution of **15** (826 mg, 922 μmol) in MeOH (5 mL) were added 10% $Pd(OH)_2$–C (420 mg) and AcOH (5 mL). The mixture was stirred for 6 h at rt under an atmosphere of hydrogen. The catalyst was filtered off, and the filtrate was concentrated in vacuo to give colorless oil. To the solution of the thus-obtained crude product in DMF (5 mL) were added $Et_3N$ (0.154 mL, 1.11 mmol) and Z-Asn-ONp (393 mg, 1.01 mmol), and the solution was stirred for 24 h at rt. The reaction mixture was concentrated in vacuo, and the residue was dissolved in $CHCl_3$ (20 mL). The solution was washed with 10% citric acid (3× 5 mL), saturated aqueous $NaHCO_3$ (3× 10 mL) and brine (3× 10 mL). The organic layer was dried over anhydrous $MgSO_4$, and concentrated in vacuo. The crude product was purified by silica-gel column chromatography (PSQ 100B, 20 g, $CHCl_3$/MeOH = 97:3). The fractions containing the desired product were combined and concentrated in vacuo to give **16** as colorless oil (866 mg, 80.1%). $^1$H NMR ($CDCl_3$) $\delta$ 1.24–1.30 (5H, m), 1.33–1.45 (36H, s), 1.65–1.89 (6H, m), 2.98–3.34 (16H, m), 4.45–4.58 (1H, m), 5.11 (2H, s), 7.34–7.39 (5H, m); MS Found *m*/*z* 1001.3. Calcd for $C_{48}H_{82}N_8O_{13}Na$: $[M+Na]^+$, *m*/*z* 1001.6.

#### 4.2.10. 4,8-Di(*tert*-butoxycarbonyl)-$N^{12}$-[(7-hydroxycoumarin-3-carbonyl)asparaginyl]-$N^1$-[$N^{\alpha}$,$N^{\varepsilon}$-di(*tert*-butoxycarbonyl)-lysyl]-4,8-diaza-1,12-dodecanediamine (17)

To a solution of **16** (94.1 mg, 96.1 μmol) in MeOH (1.5 mL) were added 10% $Pd(OH)_2$–C (50 mg) and AcOH (1.5 mL). The mixture was stirred for 2 h at rt under an atmosphere of hydrogen. The catalyst was filtered off, and the filtrate was concentrated in vacuo to give colorless oily residue. To a solution of the residue in DMF (3 mL) were added $Et_3N$ (34.0 μL, 244 μmol) and 7-hydroxycoumarin-3-carboxylic acid succinimidyl ester (Hcc-OSu) (59.2 mg, 195 μmol), and the solution stirred for 3 h at rt. The reaction mixture was diluted with $H_2O$ (5 mL), and the resulting suspension was extracted with $CHCl_3$ (3× 3 mL). The extract was washed with 10% citric acid (3× 3 mL) and brine (3× 3 mL), and dried over anhydrous $MgSO_4$, followed by concentration in vacuo. The crude product was purified by silica-gel column chromatography (PSQ 100B, 3 g, $CHCl_3$/MeOH = 97:3). The fractions containing the desired product were combined and concentrated in vacuo to give **17** as yellow oil (92.6 mg, 82.5%). $^1$H NMR ($CDCl_3$) $\delta$ 1.42 (18H, s), 1.43 (18H, s), 1.51 (8H, m4), 1.71 (6H, m), 2.78 (2H, d), 3.02–3.24 (14H, m), 4.99 (1H, t), 6.17 (1H, br s), 6.76 (1H, s), 6.78 (1H, d), 7.37 (1H, m), 8.53 (1H, s); MS Found *m*/*z* 1056.0. Calcd for $C_{50}H_{80}N_8O_{15}Na$: $[M+Na]^+$, *m*/*z* 1055.6.

#### 4.2.11. 4,8-Di(*tert*-butoxycarbonyl)-$N^{12}$-[(7-hydroxycoumarin-4-acetyl)asparaginyl]-$N^1$-[$N^{\alpha}$,$N^{\varepsilon}$-di(*tert*-butoxycarbonyl)lysyl]-4,8-diaza-1,12-dodecanediamine (18)

To a solution of **16** (94.1 mg, 0.0961 mmol) in MeOH (1.5 mL) were added 10% $Pd(OH)_2$–C (50 mg) and AcOH (1.5 mL). The mixture was stirred for 2 h at rt under an atmosphere of hydrogen. The catalyst was filtered off, and the filtrate was concentrated in vacuo to give colorless oil. To a solution of the residue in DMF (3 mL) were added $Et_3N$ (34.0 μL, 244 μmol) and 7-hydroxycoumarin-4-acetic acid succinimidyl ester (Hca-OSu) (59.2 mg, 195 μmol), and the solution stirred for 3 h at rt. The reaction mixture was diluted with $H_2O$ (5 mL), and the resulting suspension was extracted with $CHCl_3$ (3× 3 mL). The extract was washed with 10% citric acid (3× 3 mL) and brine (3× 3 mL), and the organic layer was dried over anhydrous $MgSO_4$, followed by concentration in vacuo. The crude product was purified by silica-gel column chromatography (PSQ 100B, 3 g, $CHCl_3$/MeOH = 97:3). The fractions containing the desired product were combined and concentrated in vacuo to give **18** as yellow oil (92.6 mg, 79.3%). $^1$H NMR ($CDCl_3$) $\delta$ 1.42 (18H, s), 1.43 (18H, s), 1.51 (8H, m), 1.71 (6H, m), 2.58 (2H, s), 2.78 (2H, d), 3.02–3.24 (14H, m), 4.99 (1H, t), 6.17 (1H, br s), 6.76 (1H, s), 6.78 (1H, d), 7.37 (1H, m), 8.53 (1H, s); MS Found *m*/*z* 1069.4. Calcd for $C_{50}H_{80}N_8O_{15}Na$: $[M+Na]^+$, *m*/*z* 1068.6.

#### 4.2.12. Hcc-[des-Dhpa-(NPTX-594)] (2)

To a solution of **17** (30.3 mg, 29.3 μmol) in $CH_2Cl_2$ (1.5 mL) was added TFA (3.5 mL), and the mixture was stirred for 3 h at rt. The solvent was removed in vacuo, and the thus-obtained crude product was purified by RP-HPLC. The fractions containing the desired product were combined and lyophilized to give **2** (14.6 mg, 60.1%) as yellow powdery 4 TFA salt. $^1$H NMR ($D_2O$) $\delta$ 1.34 (2H, q), 1.50 (2H, m), 1.59 (2H, m), 1.81 (6H, m), 1.99 (2H, m), 2.71–2.91 (4H, m), 3.00 (8H, m), 3.17 (2H, q, *J* = 6.6 Hz), 3.26 (2H, m), 3.84 (1H, t, *J* = 6.6 Hz), 6.72 (1H, s), 6.81 (1H, d, *J* = 8.7 Hz), 7.56 (1H, d, *J* = 8.7 Hz), 8.58 (1H, s); MS Found *m*/*z* 633.8. Calcd for $C_{30}H_{49}N_8O_7$: $[M+H]^+$, *m*/*z* 633.4.

#### 4.2.13. Hca-[des-Dhpa-(NPTX-594)] (3)

To a solution of **18** (50.0 mg, 47.7 μmol) in $CH_2Cl_2$ (1.5 mL) was added TFA (3.5 mL), and the mixture was stirred for 4 h at rt. The solvent was removed in vacuo, and the thus-obtained crude product was purified by RP-HPLC. The fractions containing the desired product were combined, and lyophilized to give **3** (29.3 mg, 67.3%) as yellow powdery 4 TFA salt. $^1$H NMR ($D_2O$) $\delta$ 1.34 (2H, q), 1.51 (2H, m), 1.58 (2H, m), 1.81 (6H, m), 1.99 (2H, m), 2.56 (2H, s), 2.71–2.91 (4H, m), 3.00 (8H, m), 3.17 (2H, q, *J* = 6.6 Hz), 3.26 (2H, m), 3.84 (1 H, t, *J* = 6.6 Hz), 6.72 (1H, s), 6.87 (1H, d, *J* = 8.7 Hz), 7.66 (1H, d, *J* = 8.7 Hz), 8.58 (1H, s); MS Found *m*/*z* 647.3. Calcd for $C_{30}H_{49}N_8O_7$: $[M+H]^+$, *m*/*z* 647.4.

#### 4.2.14. $N^{12}$-Benzyloxycarbonyl-$N^1$-[*N*-($N^4$-*tert*-butoxycarbonyl-4-aminobutyl)-*N*-(2-nitrobenzenesulfonyl)glycyl]-4,8-di(2-nitrobenzenesulfonyl)-4,8-diaza-1,12-dodecanediamine (19)

To a solution of **13** (2.43 g, 3.01 mmol) in AcOEt (20 mL) was added 4 M HCl/AcOEt (20 mL), and the solution was stirred for 1 h at rt. The solvent was removed in vacuo to obtain the residue as colorless oily residue. The solution of the thus-obtained crude product in $CH_2Cl_2$ (35 mL) was neutralized with $Et_3N$, and then Boc-Abg(Ns)-OH[20] (1.95 g, 4.52 mmol), HOBt·$H_2O$ (761 mg, 4.97 mmol), EDC·HCl (953 mg, 4.97 mmol) and $Et_3N$ (504 μL, 5.47 mmol) were added to the solution. The mixture was stirred for 20 h at rt, and then concentrated in vacuo. The residue was dissolved in AcOEt (100 mL), and the solution was washed with 10% citric acid (3× 30 mL), saturated aqueous $NaHCO_3$ (3× 30 mL) and brine (3× 30 mL). The organic layer was dried over anhydrous $MgSO_4$, and concentrated in vacuo. The thus-obtained crude prod-

uct was purified by silica-gel column chromatography (PSQ 100B, 80 g, hexane/AcOEt = 1:3). The fractions containing the desired product were combined and concentrated in vacuo to give **19** as yellow oil (2.87 g, 85.3%). $^1$H NMR ($CDCl_3$): $\delta$ 1.42 (9H, s), 1.51–1.71 (12H, m), 3.03–3.27 (16H, m), 3.95 (2H, s), 4.64 (1H, br s), 5.02 (1H, br s), 5.09 (2H, s), 6.74 (1H, br s), 7.32–7.36 (5H, m), 7.58–7.76 (9H, m), 7.95–8.07 (3H, m); MS Found *m*/*z* 1142.6. Calcd for $C_{47}H_{61}N_9O_{17}S_3Na$: $[M+Na]^+$, *m*/*z* 1142.3.

#### 4.2.15. $N^{12}$-Benzylcarbonyl-4,8-di(*tert*-butoxycarbonyl)-$N^1$-[*N*-($N^4$-*tert*-butoxycarbonyl-4-aminobutyl)-*N*-(*tert*-butoxycarbonyl)glycyl]-4,8-diaza-1,12-dodecanediamine (20)

To a suspension of **19** (1.33 g, 1.19 mmol) in $CH_3CN$ (25 mL) were added $Cs_2CO_3$ (2.32 g, 7.12 mmol) and PhSH (550 μL, 5.36 mmol). The mixture was stirred for 15.5 h at rt, and then the solvent was removed in vacuo to obtain colorless oily residue. The residue was dissolved in AcOEt (30 mL), and the solution was once dried over anhydrous $MgSO_4$, followed by concentration in vacuo. To the solution of the thus-obtained crude product in $CH_2Cl_2$ (2.5 mL) were added $Et_3N$ (0.765 mL, 5.49 mmol) and $Boc_2O$ (817 mg, 4.28 mmol), and the solution was stirred for 21 h at rt. The solvent was removed in vacuo, and the residue was dissolved in AcOEt (100 mL). The solution was washed with 10% citric acid (3× 30 mL), saturated aqueous $NaHCO_3$ (3× 30 mL) and brine (3× 30 mL). The organic layer was dried over anhydrous $MgSO_4$, and then concentrated in vacuo. The crude product was purified by silica-gel column chromatography (PSQ 100B, 20 g, benzene/AcOEt = 1:1). The fractions containing the desired product were combined and concentrated in vacuo to give **20** as colorless oil (913 mg, 76.7%). $^1$H NMR ($CDCl_3$) $\delta$ 1.44 (37H, s), 1.61–1.74 (4H, m), 2.52–2.59 (2H, m), 2.94–3.26 (18H, m),3.81 (2H, s), 4.51 (1H, br s), 4.68 (1H, br s), 5.12 (2H, s), 6.44 (1H, br s), 6.92 (1H, br s), 7.33–7.36 (5H, m); MS Found *m*/*z* 887.5. Calcd for $C_{44}H_{76}N_6O_{11}Na$: $[M+Na]^+$, *m*/*z* 887.5.

#### 4.2.16. $N^{12}$-($N^{\alpha}$-Benzyloxycarbonylasparaginyl)-4,8-di(*tert*-butoxycarbonyl)-$N^1$-[*N*-($N^4$-*tert*-butoxycarbonyl-4-aminobutyl)-*N*-(*tert*-butoxycarbonyl)glycyl]-4,8-diaza-1,12-dodecanediamine (21)

To a solution of **20** (901 mg, 1.02 mmol) in MeOH (5 mL) were added 10% $Pd(OH)_2$–C (500 mg) and AcOH (5 mL). The mixture was stirred for 6 h at rt under an atmosphere of hydrogen. The catalyst was filtered off, and the filtrate was concentrated in vacuo to obtain colorless oily residue. To the solution of the thus-obtained residue in DMF (5 mL) were added $Et_3N$ (0.154 mL, 1.11 mmol) and Z-Asn-ONp (434 mg, 1.12 mmol), and the solution was stirred for 24 h at rt. The reaction mixture was concentrated in vacuo, and the residue was dissolved in $CHCl_3$ (20 mL). The solution was washed with 10% citric acid (3× 5 mL), saturated aqueous $NaHCO_3$ (3× 10 mL) and brine (3× 10 mL). The organic layer was dried over anhydrous $MgSO_4$, and concentrated in vacuo. The crude product was purified by silica-gel column chromatography (PSQ 100B, 20 g, $CHCl_3$/MeOH = 97:3). The fractions containing the desired product were combined and concentrated in vacuo to give **21** as colorless oil (800 mg, 80.1%). $^1$H NMR ($CDCl_3$) $\delta$ 1.24–1.30 (5H, m), 1.33–1.45 (36H, s), 1.65–1.89 (6H, m), 2.98–3.34 (16H, m), 4.45–4.58 (1H, m), 5.11 (2H, s), 7.34–7.39 (5H, m); MS Found *m*/*z* 1001.3. Calcd for $C_{48}H_{82}N_8O_{13}Na$: $[M+Na]^+$, *m*/*z* 1001.6.

#### 4.2.17. 4,8-Di(*tert*-butoxycarbonyl)-$N^1$-[*N*-($N^4$-*tert*-butoxycarbonyl-4-aminobutyl)-*N*-(*tert*-butoxycarbonyl)glycyl]-$N^{12}$-[(7-hydroxycoumarin-3-carbonyl)asparaginyl]-4,8-diaza-1,12-dodecanediamine (22)

To a solution of **21** (259 mg, 265 μmol) in MeOH (5 mL) were added 10% $Pd(OH)_2$–C (150 mg) and AcOH (5 mL). The mixture was stirred for 2 h at rt under an atmosphere of hydrogen. The catalyst was filtered off, and the filtrate was concentrated in vacuo to obtain colorless oily residue. To a solution of the residue in DMF (3 mL) were added $Et_3N$ (44.3 μL, 318 μmol) and Hcc-OSu (88.4 mg, 292 μmol), and the solution was stirred for 3 h at rt. The reaction mixture was diluted with $H_2O$ (5 mL), and the resulting suspension was extracted with $CHCl_3$ (3× 3 mL). The extract was washed with 10% citric acid (3× 3 mL) and brine (3× 3 mL), and dried over anhydrous $MgSO_4$, followed by concentration in vacuo. The crude product was purified by silica-gel column chromatography (PSQ 100B, 20 g, $CHCl_3$/MeOH = 97:3). The fractions containing the desired product were combined and concentrated in vacuo to give **22** as yellow oil (226 mg, 82.5%). $^1$H NMR ($CDCl_3$) $\delta$ 1.42 (18H, s), 1.43 (18H, s), 1.51 (8H, m4), 1.71 (6H, m), 2.78 (2H, d), 3.02–3.24 (14H, m), 4.99 (1H, m), 6.17 (1H, br s), 6.76 (1H, s), 6.78 (1H, d), 7.37 (1H, m), 8.53 (1H, s); MS Found *m*/*z* 1055.9. Calcd for $C_{50}H_{80}N_8O_{15}Na$: $[M+Na]^+$, *m*/*z* 1055.6.

#### 4.2.18. 4,8-Di(*tert*-butoxycarbonyl)-$N^1$-[*N*-($N^4$-*tert*-butoxycarbonyl-4-aminobutyl)-*N*-(*tert*-butoxycarbonyl)glycyl]-$N^{12}$-[(7-hydroxycoumarin-4-acetyl)asparaginyl]-4,8-diaza-1,12-dodecanediamine (23)

To a solution of **21** (300 mg, 306 μmol) in MeOH (5 mL) were added 10% $Pd(OH)_2$–C (150 mg) and AcOH (5 mL). The mixture was stirred for 2 h at rt under an atmosphere of hydrogen. The catalyst was filtered off, and the filtrate was concentrated in vacuo to obtain colorless oily residue. To a solution of the residue in DMF (5 mL) were added $Et_3N$ (51.1 μL, 367 μmol) and 7-hydroxycoumarin-4-acetic acid succinimidyl ester (Hca-OSu) (107 mg, 337 μmol), and the solution was stirred for 3 h at rt. The reaction mixture was diluted with $H_2O$ (5 mL), and the resulting suspension was extracted with $CHCl_3$ (3× 3 mL). The extract was washed with 10% citric acid (3× 3 mL) and brine (3× 3 mL), and dried over anhydrous $MgSO_4$, followed by concentration in vacuo. The crude product was purified by silica-gel column chromatography (PSQ 100B, 20 g, $CHCl_3$/MeOH = 97:3). The fractions containing the desired product were combined and concentrated in vacuo to give **23** as yellow oil (254 mg, 79.3%). $^1$H NMR ($CDCl_3$) $\delta$ 1.42 (18H, s), 1.43 (18H, s), 1.51 (8H, m), 1.71 (6H, m), 2.58 (2H, s), 2.78 (2H, d), 3.02–3.24 (14H, m), 4.99 (1H, m), 6.17 (1H, br s), 6.76 (1H, s), 6.78 (1H, d), 7.37 (1H, m), 8.53 (1H, s); MS Found *m*/*z* 1069.4. Calcd for $C_{51}H_{82}N_8O_{15}Na$: $[M+Na]^+$, *m*/*z* 1069.6.

#### 4.2.19. Hcc,Abg-[des-Dhpa,Lys-(NPTX-594)] (4)

To a solution of **22** (50.3 mg, 48.7 μmol) in $CH_2Cl_2$ (1.5 mL) was added TFA (3.5 mL), and the mixture was stirred for 3 h at rt. The solvent was removed in vacuo, and the residue was dissolved in $H_2O$ (3 mL), followed by reconcentration in vacuo. The thus-obtained crude product was purified by RP-HPLC. The fractions containing the desired product were combined and lyophilized to give **4** (20.6 mg, 38.8%) as yellow powdery 4 TFA salt. $^1$H NMR ($D_2O$) $\delta$ 1.34 (2 H, q), 1.50 (2H, m), 1.59 (2H, m), 1.81 (6H, m), 1.99 (2H, m), 2.71–2.91 (4H, m), 3.00 (8H, m), 3.17 (2H, q, *J* = 6.6 Hz), 3.27 (2H, s), 3.84 (1H, t, *J* = 6.6 Hz), 6.72 (1H, s), 6.81 (1H, d), 7.56 (1H, m), 8.58 (1H, s); MS Found *m*/*z* 633.1. Calcd for $C_{30}H_{49}N_8O_7$: $[M+H]^+$, *m*/*z* 633.4.

#### 4.2.20. Hca,Abg-[des-Dhpa,Lys-(NPTX-594)] (5)

To a solution of **23** (50.9 mg, 48.6 μmol) in $CH_2Cl_2$ (1.5 mL) was added TFA (3.5 mL). The mixture was stirred for 4 h at rt. The solvent was removed in vacuo, and the residue was dissolved in $H_2O$ (3 mL), followed by reconcentration in vacuo. The thus-obtained crude product was purified by RP-HPLC. The fractions containing the desired product were combined and lyophilized to give **5** (32.5 mg, 60.6%) as yellow powdery 4 TFA salt. $^1$H NMR ($D_2O$) $\delta$ 1.34 (2H, q), 1.50 (2H, m), 1.59 (2H, m), 1.81 (6H, m), 1.99 (2H, m), 2.56 (2H, s), 2.71–2.91 (4H, m), 3.00 (8H, m), 3.17 (2H, m),

3.26 (2H, s), 3.84 (1H, m), 6.72 (1H, s), 6.81 (1H, d), 7.56 (1H, m), 8.58 (1H, s); MS Found *m*/*z* 647.7. Calcd for $C_{30}H_{51}N_8O_7$: $[M+H]^+$, *m*/*z* 647.4.

### 4.3. Biological assay[14,21]

Each 2 μL solution of the synthetic analogs in PBS(−) was injected between the second and third pair of legs of crickets (*Grillus bimaculatus*, 700–800 mg). In general, we first prepare 5 mM solution of the toxin, and then a portion of the solution is diluted successively to 1 mM, $1 \times 10^{-1}$ mM, $1 \times 10^{-2}$ mM, $1 \times 10^{-3}$ mM, $1 \times 10^{-4}$ mM, $1 \times 10^{-5}$ mM and $1 \times 10^{-6}$ mM. The $ED_{50}$ value of each toxin is given in nmol of toxin per g of cricket, which represents the effective dose to paralyze 50% of treated crickets at 5 min after injection. The inability of crickets to upturn when they were placed on their back was employed as the criterion for paralysis. The $ED_{50}$ value was obtained by probit analysis[22] of data from three groups of 10 crickets.

## References and notes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